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(54) TNF ligands.

(57) Ligands to a member of the TNF/NGF receptor family are provided. The ligands bind to the region of the C-terminal cysteine loop of such a receptor. A process for the preparation of the ligands is also provided, as well as pharmaceutical compositions comprising the ligands.

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FIELD OF THE INVENTION

The present invention relates to ligands to Tumor Necrosis Factor receptors (TNF-Rs) which inhibit the effect of TNF but not its binding to the TNF-Rs, as well as to ligands interacting with other receptors of the TNF/NGF receptor family.

BACKGROUND OF THE INVENTION

Tumor necrosis factor (TNF) is a pleiotropic cytokine, produced by a number of cell types, mainly by activated macrophages. It is one of the principal mediators of the immune and inflammatory response. Interest in its function has greatly increased, recently, in view of evidence of the involvement of TNF in the pathogenesis of a wide range of disease states, including endotoxin shock, cerebral malaria and graft-versus-host reaction. Since many of the effects of TNF are deleterious to the organism, it is of great interest to find ways of blocking its action on host cells. An evident target for such intervention are the molecules to which TNF has to bind in order to exert its effects, namely the TNF-Rs. These molecules exist not only in cell-bound, but also in soluble forms, consisting of the cleaved extra-cellular domains of the intact receptors (see Nophar et al., EMBO Journal, 9(10):3269-78, 1990). The soluble receptors maintain the ability to bind TNF, and thus have the ability to block its function by competition with surface receptors.

Another method of TNF inhibition based on the principle of competing with cell-bound molecules, is the use of antibodies recognizing TNF receptors and blocking the ligand binding.

The cell surface TNF-Rs are expressed in almost all cells of the body. The various effects of TNF, the cytotoxic, growth-promoting and others, are all signalled by the TNF receptors upon the binding of TNF to them. Two forms of these receptors, which differ in molecular size: 55 and 75 kilodaltons, have been described, and will be called herein p55 and p75 TNF-R, respectively. It should be noted, however, that there exist publications which refer to these receptors also as p60 and p80.

The TNF-Rs belong to a family of receptors which are involved in other critical biological processes. Examples of these receptors are the low affinity NGF receptor, which plays an important role in the regulation of growth and differentiation of nerve cells. Several other receptors are involved in the regulation of lymphocyte growth, such as CDw40 and some others. Another member of the family is the FAS receptor also called APO, a receptor which is involved in signalling for apoptosis and which, based on a study with mice deficient in its function, seems to play an important role in the etiology of a lupus-like disease. Herein, this family of receptors is called "TNF/NGF receptor family".

One of the most striking features of TNF compared to other cytokines, thought to contribute to the pathogenesis of several diseases, is its ability to elicit cell death. The cell-killing activity of TNF is thought to be induced by the p55 receptor. However, this p55 receptor activity can be assisted by the p75 receptor, through a yet unknown mechanism.

European patent publication no.s 0,398,327 and 0,412,486 disclose antibodies to the soluble TNF-Rs. These antibodies were found to recognize the soluble TNF-Rs and to inhibit the binding of TNF to the TNF-Rs on the cell surface. Monovalent F(ab) fragments blocked the effect of TNF, while intact antibodies were observed to mimic the cytotoxic effect of TNF.

SUMMARY OF THE INVENTION

The present invention provides a ligand to a member of the TNF/NGF receptor family, which binds to the region of the C-terminal cysteine loop of such a receptor.

Preferably this region includes the amino acid sequence cys-163 to thr-179 in the p75 TNF-R or a corresponding region in another member of the TNF/NGF family.

Preferably, the receptor is the TNF-R, in particular the p75 TNF-R.

One such ligand includes the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 32, shown in Fig. 11, and/or the amino acid sequence for the CDR region of the light chain of this antibody shown in Fig. 12.

Another such ligand includes the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 70 shown in Fig. 11.

Yet another such ligand includes the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 57, shown in Fig. 11.

The above antibodies are called herein, for simplicity's sake, "group 32" antibodies.

In another aspect of the invention, the ligands comprise the scFv of a group 32 antibody.

The ligands may comprise e.g. proteins, peptides, immunoadhesins, antibodies or other organic compounds.

The proteins may comprise, for example, a fusion protein of the ligand with another protein, optionally linked by a peptide linker. Such a fusion protein can increase the retention time of the ligand in the body, and thus may even allow the ligand-protein complex to be employed as a latent agent or as a vaccine.

The term "proteins" includes muteins and fused proteins, their salts, functional derivatives and active fractions.

The peptides include peptide bond replacements and/or peptide mimetics, i.e. pseudopeptides, as known in the art (see e.g. Proceedings of the 20th European Peptide Symposium, ed. G. Jung, E. Bayer, pp. 289-336, and references therein), as well as salts and pharmaceutical preparations and/or formulations which render the bioactive peptide(s) particularly suitable for oral, topical, nasal spray, ocular pulmonary, I.V. or subcutaneous delivery, depending on the particular treatment indicated. Such salts, formulations, amino acid replacements and pseudopeptide structures may be necessary and desirable to enhance the stability, formulation, deliverability (e.g. slow release, prodrugs), or to improve the economy of production, as long as they do not adversely affect the biological activity of the peptide.

Besides substitutions, three particular forms of peptide mimetic and/or analogue structures of particular relevance when designating bioactive peptides, which have to bind to a receptor while risking the degradation by proteinases and peptidases in the blood, tissues and elsewhere, may be mentioned specifically, illustrated by the following examples: Firstly, the inversion of backbone chiral centres leading to D-amino acid residue structures may, particularly at the N-terminus, lead to enhanced stability for proteolytical degradation without adversely affecting activity. An example is given in the paper "Tritriated D-ala^1-Peptide T Binding", Smith C.S. et al., Drug Development Res. 15, pp. 371-379 (1988). Secondly, cyclic structure for stability, such as N to C interchain imides and lactames (Ede et al. in Smith and Rivier (Eds.) "Peptides: Chemistry and Biology", Escom, Leiden (1991), pp. 268-270), and sometimes also receptor binding may be enhanced by forming cyclic analogues. An example of this is given in "Confirmationally restricted thymopentin-like compounds", US Pat. 4,457,489 (1985), Goldstein, G. et al. Thirdly, the introduction of ketomethylene, methylsuflide or retroinverse bonds to replace peptide bonds, i.e. the interchange of the CO and NH moieties are likely to enhance both stability and potency. An example of this type is given in the paper "Biologically active retroinverso analogues of thymopentin", Sisto A. et al in Rivier, J.E. and Marshall, G.R. (eds) "Peptides, Chemistry, Structure and Biology", Escom, Leiden (1990), pp. 722-773).

The peptides of the invention can be synthesized by various methods which are known in principle, namely by chemical coupling methods (cf. Wunsch, E: "Methoden der organischen Chemie", Volume 15, Band 1 + 2, Synthese von Peptiden, thime Verlag, Stuttgart (1974), and Barrany, G.; Marrifield, R.B.: "The Peptides", eds. E. Gross, J. Meienhofer, Volume 2, Chapter 1, pp. 1-284, Academic Press (1980)), or by enzymatic coupling methods (cf. Widmer, F. Johansen, J.T., Carlsberg Res. Commun., Vol.44, pp. 37-46 (1979), and Kullmann, W.: "Enzymatic Peptide Synthesis" CRC Press Inc. Boca Raton, Fl. (1987), and Widmer, F., Johansen, J.T. in "Synthetic Peptides in Biology and Medicines:, eds. Alitalo, K., Partanen, P., Vatieri, A., pp.79-86, Elsevier, Amsterdam (1985)), or by a combination of chemical and enzymatic methods if this is advantageous for the process design and economy.

A cysteine residue may be added at both the amino and carboxy terminals of the peptide, which will allow the cyclisation of the peptide by the formation of a di-sulphide bond.

Any modifications to the peptides of the present invention which do not result in a decrease in biological activity are within the scope of the present invention.

There are numerous examples which illustrate the ability of anti-idiotypic antibodies (anti-Id Abs) to an antigen to function like that antigen in its interaction with animal cells and components of cells. Thus, anti-Id Abs to a peptide hormone antigen can have hormone-like activity and interact specifically with a mediator in the same way as the receptor does. (For a review of these properties see: Gaulton, G.N. and Greane, M.I. 1986. Idiotypic mimicry of biological receptors, Ann. Rev. Immunol. Vol. 4, pp. 253-280; Sege K. and Peterson, P.A., 1978, Use of anti-idiotypic antibodies as cell surface receptor probes, Proc. Natl. Acad. Sci. U.S.A., Vol. 75, pp. 2443-2447).

It is expected from this functional similarity of anti-Id Ab and antigen, that anti-Id Abs bearing the internal image of an antigen can induce immunity to such an antigen. (See review in Hiernaux, J.R., 1988, Idiotypic vaccines and infectious diseases, Infect. Immun., Vol. 56, pp. 1407-1413).

It is therefore possible to produce anti-idiotypic antibodies to the peptides of the present invention which will have similar biological activity.

Accordingly, the present invention also provides anti-idiotypic antibodies to the peptides of the present invention, the anti-idiotypic antibody being capable of inhibiting TNF toxicity, but not its binding to the receptor.

The individual specificity of antibodies resides in the structures of the peptide loops making up the Complementary Determining Regions (CDRs) of the variable domains of the antibodies. Since in general the amino acid sequence of the CDR peptides of an anti-Id Ab are not identical to or even similar to the amino acid sequence of the peptide antigen from which it was originally derived, it follows that peptides whose amino acid sequence

in quite dissimilar, in certain contexts, can take up a very similar three-dimensional structure. The concept of this type of peptide, termed a "functionally equivalent sequence" or mimotope by Geyson is known. (Geyson, H.M. et al, 1987, Strategies for epitope analysis using peptide synthesis., J. Immun. Methods, Vol. 102, pp. 259-274).

Moreover, the three-dimensional structure and function of the biologically active peptides can be simulated by other compounds, some not even peptidic in nature, but which nevertheless mimic the activity of such peptides. This field is summarized in a review by Goodman, M. (1990), (Synthesis, Spectroscopy and computer simulations in peptide research, Proc. 11th American Peptide Symposium published in Peptides-Chemistry, Structure and Biology, pp. 3-29; Eds. Rivier, J.E. and Marshall, G.R. Publisher Escom).

It is also possible to produce peptide and non-peptide compounds having the same three-dimensional structure as the peptides of the present invention. These "functionally equivalent structures" or "peptide mimics" will react with antibodies raised against the peptide of the present invention and may also be capable of inhibiting TNF toxicity.

Accordingly, a further embodiment of the present invention provides a compound the three-dimensional structure of which is similar as a pharmacophore to the three-dimensional structure of the peptides of the present invention, the compound being characterized in that it reacts with antibodies raised against the peptides of the present invention and that the compound is capable of inhibiting TNF toxicity.

More detail regarding pharmacophores can be found in Bolin et al., p. 150, Polinsky et al., p. 287, and Smith et al., p. 485, in Smith and Rivier (eds.) "Peptides: Chemistry and Biology", Escom, Leiden (1991).

All of the molecules (proteins, peptides, etc.) may be produced either by conventional chemical methods, as described herein, or by recombinant DNA methods.

The invention also provides DNA molecules encoding the ligands according to the invention, vectors containing them and host cells comprising the vectors and capable of expressing the ligands according to the invention.

The host cell may be either prokaryotic or eukaryotic.

The invention further provides DNA molecules hybridizing to the above DNA molecules and encoding ligands having the same activity.

The invention also provides pharmaceutical compositions comprising the above ligands which are useful for treating diseases induced or caused by the effects of TNF, either endogenously produced or exogenously administered.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a diagrammatic illustration of the bacterial constructs used for determining the sequence to which antibodies of the 32 group bind.

Figure 2 shows an example of the Western blotting analysis technique by which the binding of the antibodies to the constructs shown in Figure 1 have been determined.

Figures 3 & 4 show the competition of synthetic peptides whose sequences contain the region of the epitope recognized by the monoclonal antibodies of the 32 group, or parts of it, with the binding of an antibody of this group to a construct comprising part of TBP-II in which this epitope is present.

Figure 5 shows the nucleotide and deduced amino acid sequences of the p75 receptor. TBP-II and transmembranal domains are boxed and shaded. The region recognized by the group 32 antibodies is underlined.

Figure 6 shows the pattern of protection of HeLa p75.3 cells (as hereinafter defined) from TNF cytotoxicity by different monoclonal antibodies against p75 TNF-R, and fragments thereof.

Figure 7 shows the effects of a monoclonal antibody against TBP-I and several against TBP-II on the extent of killing of U937 cells by TNF.

Figures 8a and 8b show the effects of monoclonal antibody 70 and Fab fragments thereof on the binding of TNF to HeLa p75.3 cells and U937 cells, respectively.

Figure 9 shows a comparison of the effects of the antibody 32 with other antibodies against the p75 TNF-R on TNF binding to HeLa p75.3 cells.

Figure 10 shows dissociation of TNF from HeLa p75.3 cells in the presence and absence of antibody no. 70 and its monovalent Fab fragment.

Figure 11 shows the nucleotide and deduced amino acid sequences for the CDR region of the heavy chains of three monoclonal antibodies of the 32 group.

Figure 12 shows the nucleotide and deduced amino acid sequences for the CDR region of the light chains of monoclonal antibody No. 32.

Figure 13 shows the sequence homology between several members of the TNF/NGF receptor family.

DETAILED DESCRIPTION OF THE INVENTION

TNF, as stated above, is a cytokine which initiates its effect on cell function by binding to two specific cell surface receptors: the p55 and p75 receptors. Binding of antibodies to the extracellular domain of these receptors can interfere with its

effect. However, as shown in a number of studies, antibodies binding to the extracellular domain of the receptors can also trigger the effects of TNF by inducing aggregation of the p55 receptors, as well as by inducing aggregation of the p75 receptors. (Engelmann, et al. J. Biol. Chem., Vo. 265, No. 24, pp. 14497-14504, 1990; and unpublished data).

We have found that certain antibodies binding to one particular region in the p75 receptor are not mimetic but rather inhibitory to the signalling for the cytocidal effect by this receptor. This, in spite of the fact that when binding to this region, these antibodies do not block TNF binding, but rather increase it to some extent.

The present invention reveals that this region recognized by these antibodies which we call the 32 group, is the region extending between the two C-terminal cysteines in the extracellular domain of the p75 receptor, plus an additional amino acid, thr179. This region for simplicity's sake, is called "cysteine loop" throughout this specification.

The present invention also provides the nucleotide sequences and deduced amino acid sequences in the CDR of the heavy chain of the three antibodies belonging to this group, named 32, 57 and 70. A remarkable similarity between the sequence of amino acids in the CDR of the heavy chain of the 32 and 70 antibodies was found, indicating that the sequence of amino acids in the CDR of the heavy chain of these two antibodies is close to the optimum necessary for binding to the antigen. In addition, the invention also provides the nucleotide sequence and the deduced amino acid sequence of the light chain of antibody 32. Based on these sequences, small molecular weight compounds, peptides or mimetic compounds which will inhibit the function of the p75 receptors can be defined.

In evidence that such small compounds can indeed achieve this and that there is no need for aggregation of receptors, which antibodies are known to be able to do, it was found that also F(ab) monovalent fragments of the antibodies of the 32 group inhibit signalling for toxicity by the p75 receptor when they are triggered by TNF.

In view of these findings, as well as the close similarity of the receptors in this particular family, this invention relates also to agents which bind to the C-terminal cysteine loop in the extracellular domain of the various other members of the TNF/NGF receptor family and modulate the function of the other receptors, similarly to the modulation of the function of TNF. In this receptor family, the localization of cysteine in the extracellular domain and the spacing is highly conserved. Certain members of this family, e.g. CDw40, exhibit particularly high similarity to the p75 receptor. Particularly in such receptors, agents binding to these regions are expected to have effects similar to the effect of the 32 antibodies on the p75 receptor.

As stated above, the ligands according to the invention may comprise proteins, peptides, immunoadhesins, antibodies or other organic compounds.

Proteins may be isolated from cellular extracts, e.g. by ligand affinity purification employing a molecule having an amino acid sequence substantially corresponding to the above-mentioned stretch as ligand.

Peptides may be prepared by synthetizing first target peptides which correspond to the amino acid stretch of the TNF-R found in accordance with the invention to bind the ligands which inhibit the effects of TNF. Thereafter, peptide libraries are screened for other ligands which bind thereto. The peptides which bind to these regions are further screened for those which also bind to TNF-R. Finally, the peptides capably of high affinity binding with both the target peptides and the TNF-R, are screened for the ability of the peptide to perform the desired biological activity.

In a similar manner, a variety of organic molecules, including drugs known for other indications, are screened for their ability to bind to the amino acid stretch found to be critical for inhibiting the effects of TNF.

In addition to the organic molecules, also broth of biological matter such as bacteria culture products, fungi culture products, eukaryotic culture products and crude cytokine preparations are screened with the amino acid target peptides described above. Molecules obtained by this screening are then further screened for their ability to perform the desired biological function.

Alternatively, molecules are designed which spatially fit the quaternary structure of the amino acid stretch in the receptor.

The active molecules obtained by the above procedures, inasfar as they are biological substances, can also be prepared by biotechnological approaches. In this way, massive production of these molecules will be made possible. Peptides may either be produced by known peptide synthesis methods or using expression vectors containing DNA sequences encoding them. Other molecules, if produced in an enzymatic way, can be made by producing the enzymes involved in the appropriate cultured cells.

Pharmaceutical compositions containing the ligands of the present invention may be employed for antagonizing the effects of TNF in mammals.

Such compositions comprise the ligands according to the invention as their active ingredient. The pharmaceutical compositions are indicated for conditions such as septic shock, cachexia, graft-versus-host reactions, autoimmune diseases such

as rheumatoid arthritis, and the like. They are also indicated for counteracting e.g. an overdose of exogenously administered TNF.

The pharmaceutical compositions according to the invention are administered depending on the condition to be treated, via the accepted ways of administration. For example, in the case of septic shock, intravenous administration will be preferred. The pharmaceutical compositions may also be administered continuously, i.e. by way of infusion, or orally. The formulation and dose will depend on the condition to be treated, the route of administration and the condition and the body weight of the patient to be treated. The exact dose will be determined by the attending physician.

The pharmaceutical compositions according to the invention are prepared in the usual manner, for example by mixing the active ingredient with pharmaceutically and physiologically acceptable carriers and/or stabilizers and/or excipients, as the case may be, and are prepared in dosage form, e.g. by lyophilization in dosage vials.

As used herein the term "muteins" refers to analogs of the proteins, peptides and the like in which one or more of the amino acid residues of the protein found to bind are replaced by different amino acid residues or are deleted, or one or more amino acid residues are added to the original sequence, without changing considerably the activity of the resulting product. These muteins are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable therefor.

The term "fused protein" refers to a polypeptide comprising the ligands or a mutein thereof fused with another protein which has an extended residence time in body fluids. The ligands may thus be fused to another protein, polypeptide or the like, e.g. an immunoglobulin or a fragment thereof.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the ligands, muteins and fused proteins thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid.

"Functional derivatives" as used herein cover derivatives of the ligands and their fused proteins and muteins, which may be prepared from the functional groups which occur as side chains on the residues or the N- or C- terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the ligand and do not confer toxic properties on compositions containing it. These derivatives may, for example, include polyethylene glycol side-chains which may mask antigenic sites and extend the residence of the ligands in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl groups (for example that of seryl or threonyl residues) formed with acyl moieties.

The invention is illustrated by the following non-limiting examples:

EXAMPLE 1: Determination of the region of the p75 receptor which is recognized by the group 32 antibodies

In example 5 of the main application, a number of constructs were prepared by expression in E. coli, and it was concluded that the epitope recognized by antibody no. 32 maps between amino acids 125-182.

We have now prepared further constructs and the complete list of constructs examined, as well as their relationship to the structure of the soluble p75R are shown in Fig. 1. Constructs recognized by the antibodies of the 32 group are listed in bold numbers and illustrated as solid lines. Those not reacting with these antibodies are listed in thin numbers and illustrated by broken lines. All constructs are identified by their N- and C-terminal amino acid residues.

Figure 1, above the diagrammatic illustration of the constructs, shows the amino acid sequence of part of the p75 TNF-R, the regions corresponding to the soluble form of the receptor and the transmembranal region being boxed. Amino acid residues conserved between man and mouse are underlined.

The Western blotting analysis shown in Figure 2, of the binding of the group 32 antibodies to some of the constructs shown in Figure 1 was carried out as in Example 5 of the main application.

EXAMPLE 2: Competition for binding to the extracellular domain of the p75 TNF-R between group 32 antibodies and synthetic peptides

A number of synthetic peptides whose sequences correspond to various parts of the region on the TNF-R suspected to be the group 32 epi-

tope were synthetized (residues 160-179, 162-179, 163-179, 165-179 and 167-179). The peptides were examined in an ELISA test for their ability to compete for the binding to the antibodies of the 32 group.

A bacterially produced construct corresponding to amino acids 3 to 180 of the p75 TNF-R (p75 construct in Fig. 3) was applied, at the indicated concentrations, to PVC plates precoated with antibody 32 followed by application of rabbit antiserum to TBP-II (p75 soluble TNF-R). The amount of rabbit antiserum bound to the plate was determined by applying goat antiserum against rabbit immunoglobulin, coupled to horse-radish peroxidase and enzymatic assessment of the amount of goat immunoglobulin bound to the plate. Figure 3 shows the data of an experiment in which a synthetic peptide corresponding to amino acid residues 163 to 179 was found to compete for the binding.

Figure 4 shows the data of an experiment in which a fusion protein of maltose binding protein (MBP) with the sequence of amino acids extending from 125 to 192 of the p75 receptor was used to coat PVC plates at a concentration of 10µg/ml, then the No. 32 McAb was applied at a concentration of 2µg/ml together with the indicated concentrations of different peptides:

DW16 - amino acids 165-179
DW18 - amino acids 163-179
DW19 - amino acids 162-179
DW21 - amino acids 160-179

Thereafter, the reaction was developed by adding goat anti-mouse coupled to horseradish peroxidase. As shown in Fig. 4, marked inhibition of fusion protein recognition by monoclonal antibody No. 32 was observed only with the three peptides covering the whole epitope.

EXAMPLE 3: Mutational study of the 32 epitope

Replacing cysteine 178 with alanine in a recombinant peptide whose sequence corresponds to amino acids 3 to 181, made this protein unrecognizable by the 32 group antibodies. This finding suggests that in order to be recognized by these antibodies, the two cysteines in the group 32 epitope region must be free to interact with each other; i.e. that the structure recognized by the antibodies is a loop. In support of this notion, we found that reduction of the peptide with dithiothreithol prior to SDS PAGE and Western blotting analysis somewhat decreased the effectivity of its recognition by the 32 group antibodies, and reduction by dithiothreithol followed by alkylation with iodoacetimide made it completely unrecognizable by the antibodies.

EXAMPLE 4: Effects of various antibodies and fragments thereof on TNF toxicity

(a) In order to compare the function of the 32 group antibodies, not only to antibodies which bind to the receptor upstream to the 32 epitope region (as most of the anti-TBP-II antibodies are expected to), but also to antibodies that bind to the receptor downstream to that epitope region, we immunized mice with a chimeric construct corresponding to the region extending downstream to the 32 epitope (amino acids 181 to 235; the "stalk" region), linked to MBP. The rabbits developed antibodies which bound to the chimera with which they were immunized as well as to the intact p55 TNF receptor. These antibodies were affinity purified by binding to the chimeric protein, linked to an Affi-gel 10 column, and tested for effect on TNF function and binding. (The affinity purified antibody preparation was termed "318").

(b) All monoclonal anti-TBP-II antibodies as well as the affinity purified antistalk antibodies were tested for effect on TNF toxicity in clones of the epitheloid HeLa cells which were made to overexpress the p75 receptors (by their transfection with the p75 receptor's cDNA. We called the particular over-expressing clone used in the experiments presented here, HeLa p75.3). The only antibodies found to inhibit TNF function were the antibodies of the group 32 epitope; that, in spite of the fact that they do not inhibit, but somewhat increase TNF binding to the receptor (Figs. 8 and 9). Two of the other anti-TBP-II antibodies (No. 67 of Figs. 6 and 9 and number 81) had very little effect on TNF binding to the receptor or on TNF toxicity. All other monoclonal anti-TBP-II antibodies somewhat potentiated the cytocidal effect of TNF even though competing with TNF binding (e.g. antibody 36 of Figs 6 and 9). The "anti-stalk" antibodies had very little effect on TNF binding or function (Figs. 6 and 9). Applying the anti-stalk antibodies on the cells together with antibodies of the 32 group did not interfere with the inhibitory effect of the latter on TNF function.

(c) The same panel of antibodies was tested for effect on the killing of the myelocytic U937 cells by TNF. As opposed to the mimetic effect of anti-TNF receptor antibodies in the HeLa cells, neither anti-p55 nor anti-p75 receptor antibodies were found to be mimetic to the cytocidal effect of TNF on the U937 cells under the conditions of the experiment carried out. Having no ability to mimic the effect of TNF, all monoclonal antibodies which compete for TNF binding either to the p75 receptor, (e.g. antibodies 14, 31 and 36 of Figure 9) or to the p55 receptor (e.g. antibody

number 18 of Figure 7) are inhibitory to the TNF effects. Antibodies which had no effect on TNF binding to the receptors (e.g. number 67 of Figure 9) had no effect on TNF function (Figure 6). The 32 group antibodies were unique in having an ability to inhibit TNF function in this cell without having any inhibitory effect on TNF binding. The antibodies actually enhanced the binding of TNF to these cells, much more so than in the HeLa p75.3 cells (Figure 8). The inhibitory effect of the 32 group antibodies was additive to that of antibodies which block TNF binding to the p55 receptor (e.g. the combination 18/32 in Figure 7).

EXAMPLE 5: Effect of group 32 antibodies and Fab monovalent fragments thereof on the dissociation of TNF from the TNF-Rs

In order to explore the mechanism by which the 32 group antibodies cause an increase in TNF binding, we compared the rate of TNF dissociation from HeLa p75.3 cells in the presence and absence of these antibodies.

Radiolabelled TNF was added to confluent HeLa p75.3 cells and the cells were incubated for 2 hr on ice. Unbound ligand was washed away and 1 ml of binding buffer containg 500 ng/ml of cold TNF was applied into quadruplicate wells for the indicated periods of time on ice. Thereafter, the wells were washed once again with cold PBS, and amount of residual ligand was determined by measuring radioactivity of cells detached from plates by incubation with PBS/EDTA solution. The antibodies were applied throughout the assay at a concentration of 10μg/ml.

As illustrated in Fig. 10, both these antibodies as well as their F(ab) monovalent fragments caused a decrease in the rate of TNF dissociation from the receptors. Besides providing a possible explanation for the way in which these antibodies affect TNF binding to its receptors, this finding indicated an additional application for this effect. Soluble forms of the p75 TNF-Rs or of the p55 receptor or of any other member of the TNF/BGF receptor family in which a conformational change as that imposed by the 32 group antibody will occur, will serve as better inhibitors of the respective agonist.

EXAMPLE 6: Determination of nucleotide sequences and deduced amino acid sequences in the CDR of the heavy chains of monoclonal antibodies 32, 57 and 70 (Group 32 antibodies) and in the CDR of the light (Kappa) chain of antibody 32

In order to determine the nucleotide sequences of the CDR of the heavy chains of antibodies 32, 57 and 70, total RNA was isolated by the Promega protocol from the respective hybridoma cells, with the use of guanidinium thioisocyanate. First strand cDNA synthesis on this RNA was performed with the use of AMV reverse transcriptase and either oligo(dT)15-18 or an oligonucleotide complementary to the constant region of the heavy chain of murine IgG as a primer. The cDNA was used as a template for PCR, applying a partially degenerate 5'-Primer. 40 cycles of PCR were carried out. PCR products with the size of about 350 bp were purified electrophoretically and cloned into the Bluescript vector. Clones having inserts of the right size were sequenced. Double-stranded cDNA of the CDR region of the light chain of antibody no. 32 was synthesized in a similar manner.

The nucleotide sequences obtained by the dideoxy chain termination method, and the amino acid sequences deduced therefrom are shown in Figures 11 and 12. The CDR1, 2 and 3 regions are underlined.

EXAMPLE 7: Preparation of scFv of the 32 group antibodies

The cloned variable regions of the heavy and light chains of the monoclonal antibodies of the 32 group are linked with a linker of 15 amino acid length and introduced into a commercial expression vector. The vector contains a promoter, e.g. lac, a leader sequence e.g. pel-B, as well as a sequence encoding a small peptide ("tag" peptide) against which a monoclonal antibody is commercially available. The plasmid is now introduced into E. coli and the bacteria are grown to O.D. 0.5-1.0. Expression of scFv is induced by addition of IPTG and growth is continued for another 6-24 hrs. The soluble scFv-tag complex is then isolated from the culture medium by immunoaffinity purification using the monoclonal antibody against the tag and then purified on a metaloaffinity column.

Any scFv accumulating within the bacteria is purified by isolating and repeatedly washing the inclusion bodies, followed by solublization by e.g. urea or guanidinium and subsequent renaturation.

Alternative possibilities are employing an oligohistidine as the tag, using a stronger promoter instead of lac, i.e. T7, constructing the vector without the leader sequence or introducing a sequence encoding a "tail" of irrelevant sequences into the vector at the 5' end of the scFv. This "tail" should not be biologically active, since its only purpose is the creation of a longer molecule than the native scFv, thus causing a longer retention time in the body.

EXAMPLE 8:

Figure 13 shows the internal cysteine rich repeats in the extracellular domains of the two TNF-Rs and their alignment with the homologous repeats in the extracellular domain of the human FAS, nerve growth factor receptor (NGF) and CDw40, as well as rat Ox40. The amino acid sequences (one letter symbols) are aligned for maximal homology. The positions of the amino acids within the receptors are denoted in the left hand margin.

EXAMPLE 9: Creation of recombinant DNA molecules comprising nucleotide sequences coding for the active peptides and other molecules and their expression

The peptides and other molecules can also be prepared by genetic engineering techniques and their preparation encompasses all the tools used in these techniques. Thus DNA molecules are provided which comprise the nucleotide sequence coding for such peptides and other biological molecules. These DNA molecules can be genomic DNA, cDNA, synthetic DNA and a combination thereof.

Creation of DNA molecules coding for such peptides and molecules is carried out by conventional means, once the amino acid sequence of these peptides and other molecules has been determined.

Expression of the recombinant proteins can be effected in eukaryotic cells, bacteria or yeasts, using the appropriate expression vectors. Any method known in the art may be employed.

For example, the DNA molecules coding for the peptides or other molecules obtained by the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Maniatis, T. et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (1982)). Double-stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques.

DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing a desired biological substance, i.e. a peptide or protein (hereinafter "protein", for simplicity's sake), an expression vector should comprise also specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding for the desired protein in such a way as to permit gene expression and production of the protein. First, in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters). They are different for prokaryotic and eukaryotic cells.

The promoters that can be used in the present invention may be either constitutive, for example, the int promoter of bacteriophage lambda, the bla promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pPR325, etc., or inducible, such as the prokaryotic promoters including the major right and left promoters of bacteriophage lambda (P_L and P_R), the trp, recA, lacZ, lacI, ompF and gal promoters of E. coli, or the trp-lac hybrid promoter, etc. (Glick, B.R. (1987) J.Ind.Microbiol., 1:277-282).

Besides the use of strong promoters to generate large quantities of mRNA, in order to achieve high levels of gene expression in prokaryotic cells, it is necessary to use also ribosome-binding sites to ensure that the mRNA is efficiently translated. One example is the Shine-Dalgarno (SD) sequence appropriately positioned from the initiation codon and complementary to the 3'-terminal sequence of 16S RNA.

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of Herpes virus, the SV40 early promoter, the yeast ga14 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the peptides or other molecules of the invention and the operably linked transcriptional and translational regulatory signals is inserted into a vector which is capable of integrating the desired gene sequences into the host cell chromosome. The cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotropic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or intro-

duced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., (1983) Mol. Cell Biol., 3:280.

In a preferred embodiment, the introduced DNA molecule will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors include plasmids such as those capable of replication in E. coli, for example, pBR322, ColEI, pSC101, pACYC 184, etc. (see Maniatis et al., (1982) op. cit.); Bacillus plasmids such as pC194, pC221, pT127, etc. (Gryczan, T., The Molecular Biology of the Bacilli, Academic Press, NY (1982)); Streptomyces plasmids including plJ101 (Kendall, K.J. et al., (1987) J.Bacteriol. 169:4177-83); Streptomyces bacteriophages such as ϕC31 (Chater, K.F. et al., in: Sixth International Symposium on Actinomycetales Biology, (1986)), and Pseudomonas plasmids (John, J.F., et al. (1986) Rev.Infect.Dis. 8:693-704; and Izaki, K. (1978) Jpn. J. Bacteriol., 33:729-742).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al. (1982) Miami Wint. Symp. 19, pp. 265-274; Broach, J.R., in: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 445-470 (1981); Broach, J.R., (1982) Cell, 28:203-204; Bollon, D.P., et al. (1980) J. Clin. Hematol. Oncol., 10:39-48; Maniatis, T., in: Cell Biology: A Comprehensive Treatise, Vol. 3: Gene Expression, Academic Press, NY, pp. 563-608 (1980)).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells to be used in this invention may be either prokaryotic or eukaryotic. Preferred prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, etc. The most preferred prokaryotic host is E. coli. Bacterial hosts of particular interest include E. coli K12 strain 294 (ATCC 31446), E. coli X1776 (ATCC 31537), E. coli W3110 (F^-, lambda$^-$, prototropic (ATCC 27325)), and other enterobacteria such as Salmonella typhimurium or Serratia marcescens and various Pseudomonas species. Under such conditions, the protein will not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

Preferred eukaryotic hosts are mammalian cells, e.g. human, monkey, mouse and chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e. pre-peptides).

After the introduction of the vector, the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

Purification of the recombinant proteins is carried out by any one of the methods known for this purpose.

Deposit Information

Hybridoma TBP-II 70-2 was deposited with the Collection National de Cultures de Microorganismes, Institut Pasteur (CNCM) on March 12, 1990 and assigned No. I-928.

Hybridoma TBP-II 32-5 was deposited with the CNCM on September 1, 1993 and assigned No. I-1358.

The deposited hybridomas TBP-II 70-2 and TBP-II 32-5 are sub-clones of hybridomas TBP-II 70 and TBP-II 32, respectively and have identical properties.The production and cloning of these hybridomas has been described in EP-A 398 327.

Claims

1. A ligand to a member of the TNF/NGF receptor family which binds to the region of the C-terminal cysteine loop (as herein defined) of such a receptor.

2. A ligand according to claim 1, wherein the cysteine loop includes the amino acid sequence cys-163 to thr-179 in the p75 TNF-R, or a corresponding region in another member of the TNF/NGF receptor family.

3. A ligand according to claim 2, which comprises a ligand to a TNF-R.

4. A ligand according to any one of claims 1 to 3, wherein the receptor is the p75 TNF-R.

5. A ligand according to any one of claims 1 to 4, including the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 32 shown in Fig. 11.

6. A ligand according to any one of claims 1 to 4, including the amino acid sequence for the CDR region of the light chain of monoclonal antibody no. 32 shown in Fig. 12.

7. A ligand according to claim 5, further including the amino acid sequence in the CDR region of the light chain of monoclonal antibody no. 32, shown in Fig. 12.

8. A ligand according to any one of claims 1 to 4, including the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 70 shown in Fig. 11.

9. A ligand according to any one of claims 1 to 4, including the amino acid sequence for the CDR region of the heavy chain of monoclonal antibody no. 57 shown in Fig. 11.

10. A ligand according to any one of claims 1 to 4, including the amino acid sequence of an antibody raised against the C-terminal loop of a member of the TNF/NGF receptor family.

11. A ligand according to any one of claims 1 to 4, comprising the scFv of a group 32 antibody.

12. A ligand according to any one of claims 1 to 11, comprising a protein.

13. A ligand according to any one of claims 1 to 11, comprising a peptide.

14. A ligand according to any one of claims 1 to 11, the three-dimensional structure of which is similar as a pharmacophore to the three-dimensional structure of the protein or peptide as claimed in claims 12 and 13, and being capable of inhibiting the effect of TNF but not its binding to the TNF-R.

15. A DNA molecule encoding a ligand according to any one of claims 1 to 14, capable of expressing such a ligand.

16. A DNA molecule hybridizing to a DNA molecule according to claim 15 and capable of expressing a ligand according to any one of claims 1 to 14.

17. A replicable expression vehicle comprising a DNA molecule according to claim 15 or 16, and capable, in a transformant host cell, of expressing a ligand according to any one of claims 1 to 14.

18. A host cell transformed with the replicable expression vehicle of claim 17.

19. A host cell according to claim 18 which is a prokaryotic cell.

20. A host cell according to claim 18 which is a eukaryotic cell.

21. A process for the production of a recombinant ligand according to any one of claims 1 to 14, comprising culturing a transformed host cell according to any one of claims 18 to 20 and recovering the recombinant ligand.

22. An anti-idiotypic antibody to a ligand according to any one of claims 1 to 14, capable of inhibiting the effect of TNF, but not its binding to the TNF-R.

23. A pharmaceutical composition comprising a ligand according to any one of claims 1 to 14 and/or an anti-idiotypic antibody according to claim 22.

24. Use of a ligand according to any one of claims 1 to 14 or an anti-idiotypic antibody according to claim 22 for increasing the inhibitory effect of a soluble receptor of the TNF/NGF receptor family.

25. Use of soluble mutated receptor forms of the TNF/NGF receptor family in which the region corresponding to the group 32 epitope has the conformation occurring when antibodies of the 32 group in the p75-R bind to it, as a more effective inhibitor than the natural form for the function of the respective ligand.

26. A DNA molecule encoding a ligand of any one of claims 1 to 14.

27. A mutein or functional derivative to a ligand of any one of claims 1 to 14 or to the antibody of claim 22.

28. A fused protein comprising a ligand according to anyone of claims 1 to 14 or an antibody according to claim 22.

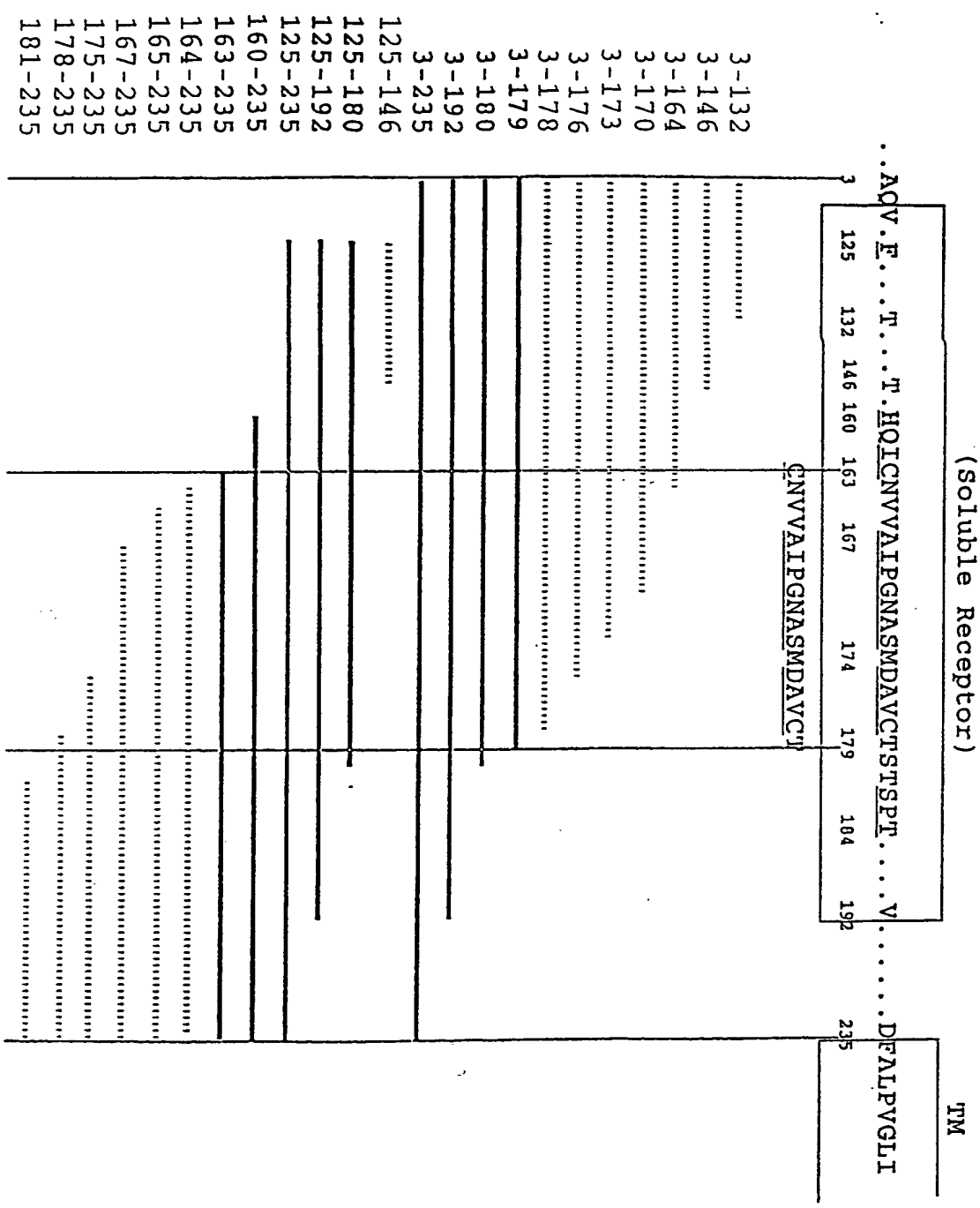

(Soluble Receptor)
TM
..AQV.E...T...T.HQICNVVAIPGNASMDAVCTSTSPT....V.....DFALPVGLI
125 132 146 160 163 167 174 179 184 192 235
CNVVAIPGNASMDAVC
3-132
3-146
3-164
3-170
3-173
3-176
3-178
3-179
3-180
3-192
3-235
125-146
125-180
125-192
125-235
160-235
163-235
164-235
165-235
167-235
175-235
178-235
181-235

FIGURE 1

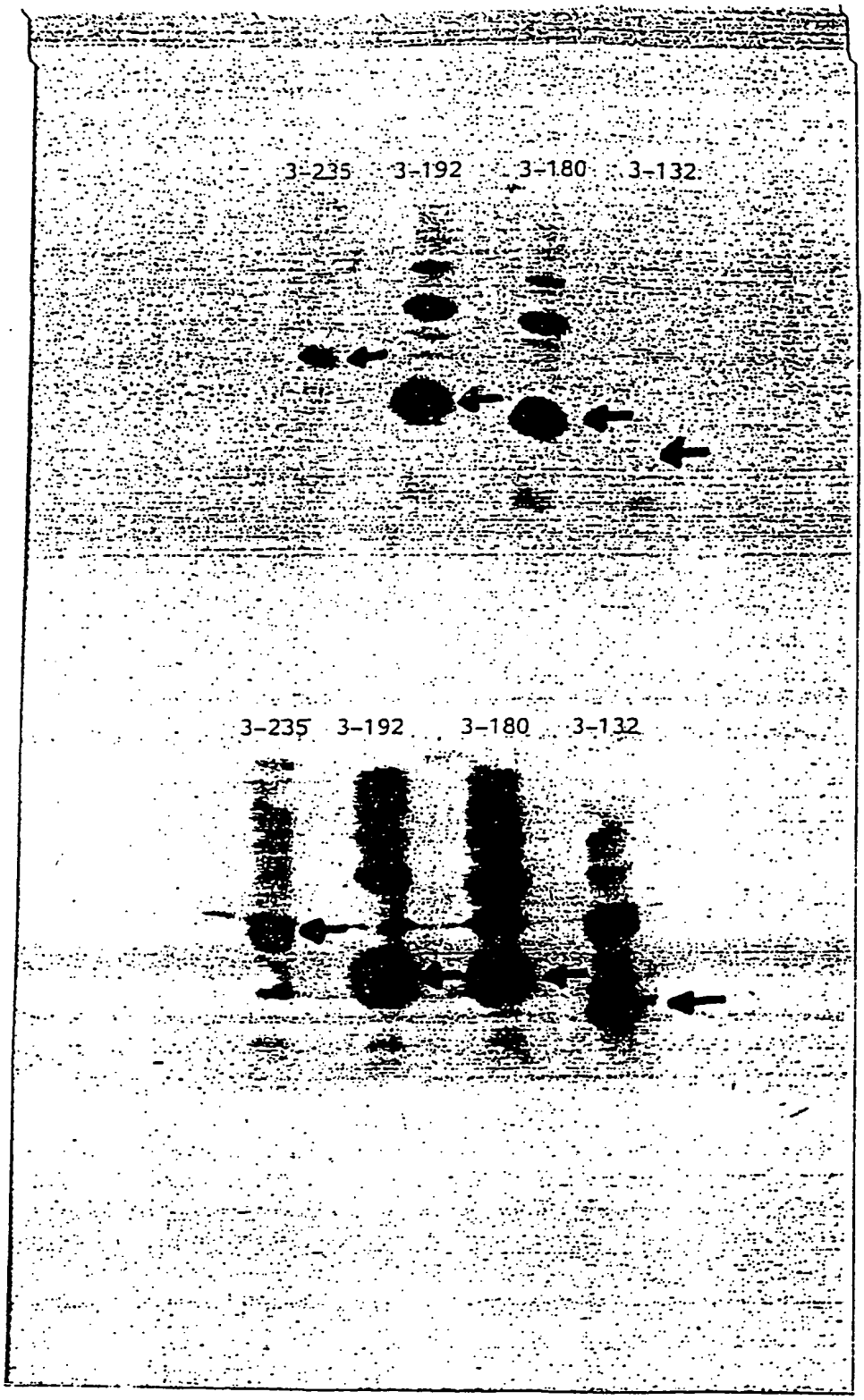


FIGURE 2

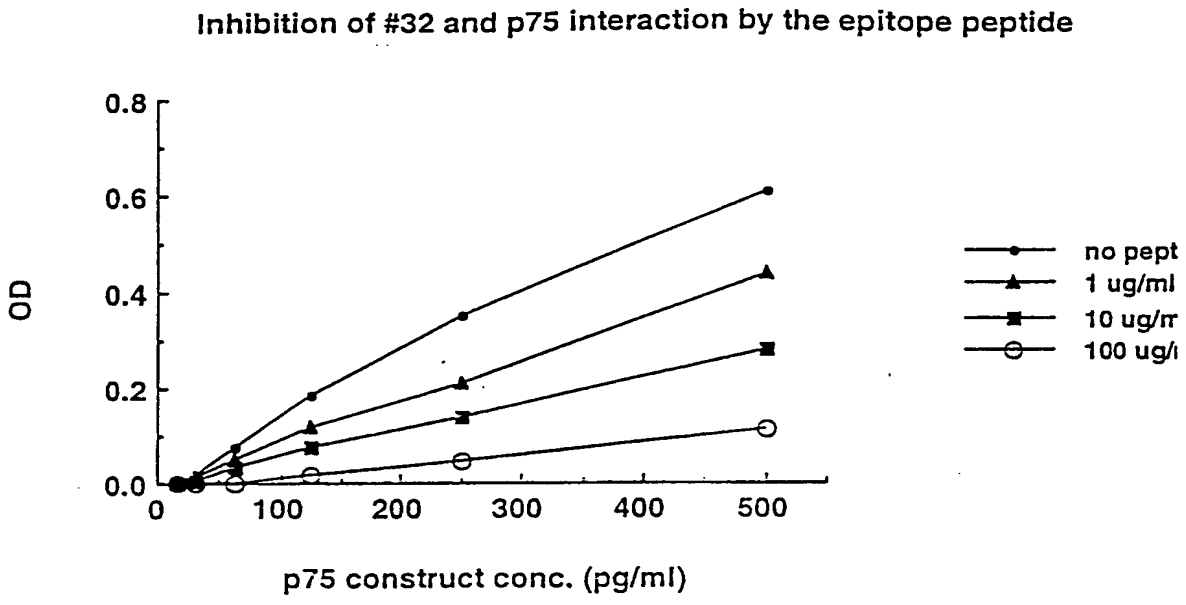

Inhibition of #32 and p75 interaction by the epitope peptide
OD
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
p75 construct conc. (pg/ml)
no pept
1 ug/ml
10 ug/m
100 ug/

FIGURE 3

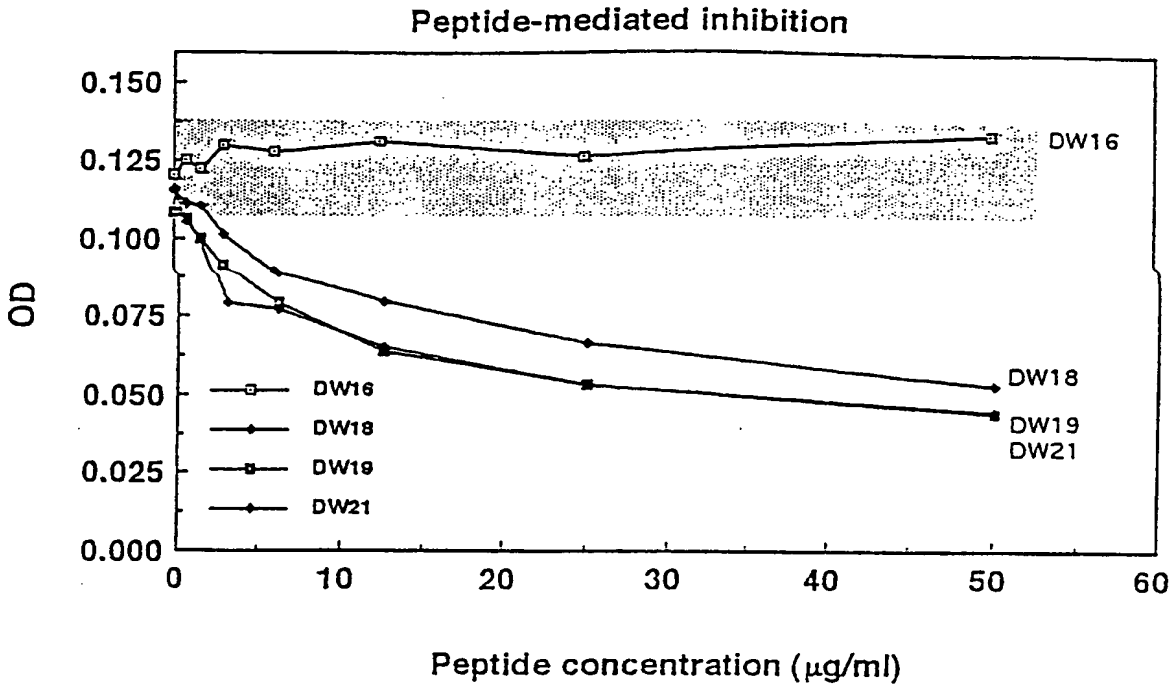

Peptide-mediated inhibition
OD
0.150
0.125
0.100
0.075
0.050
0.025
0.000
0
10
20
30
40
50
60
DW16
DW18
DW19
DW21
DW16
DW18
DW19
DW21
Peptide concentration (μg/ml)

FIGURE 4

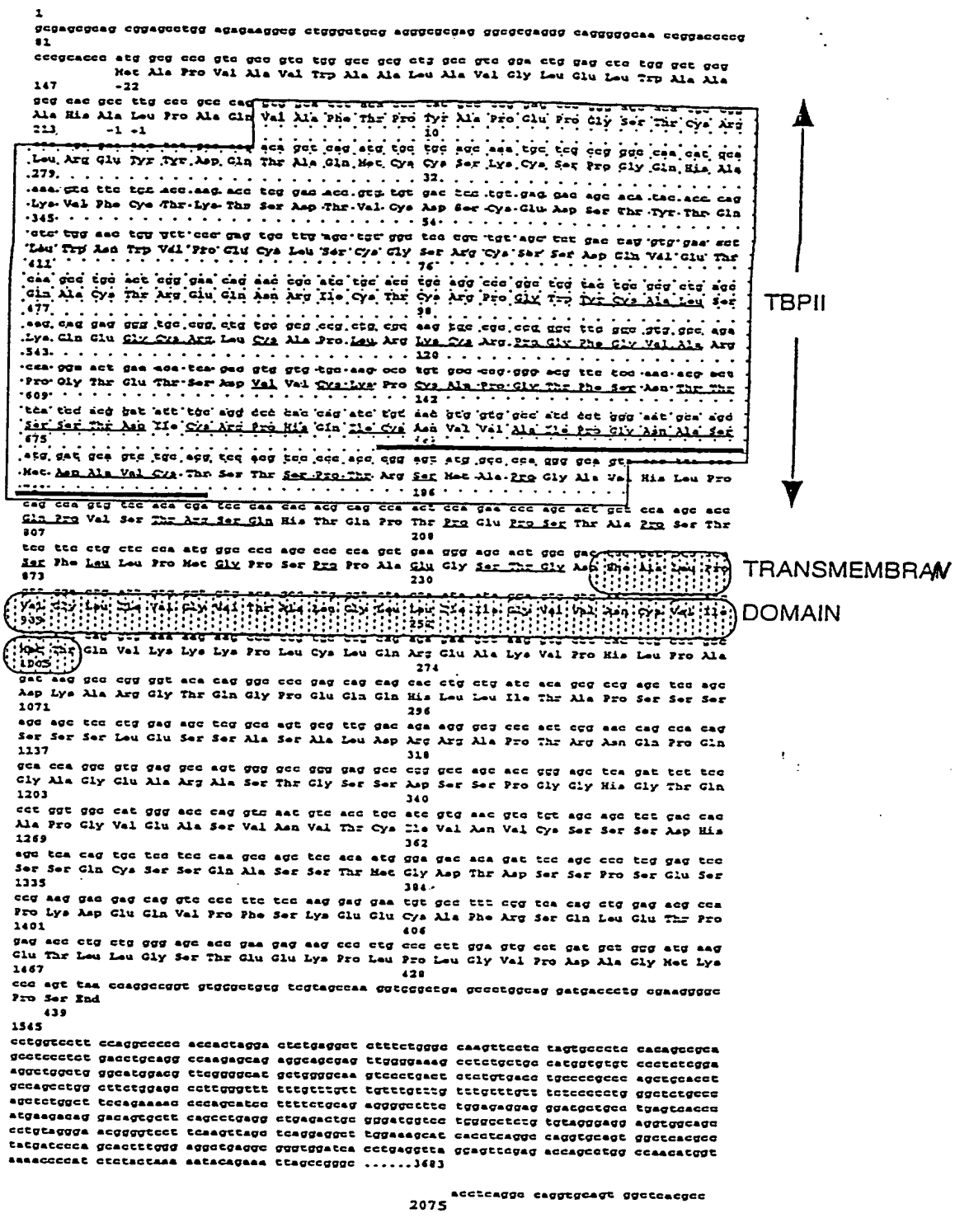


FIGURE 5

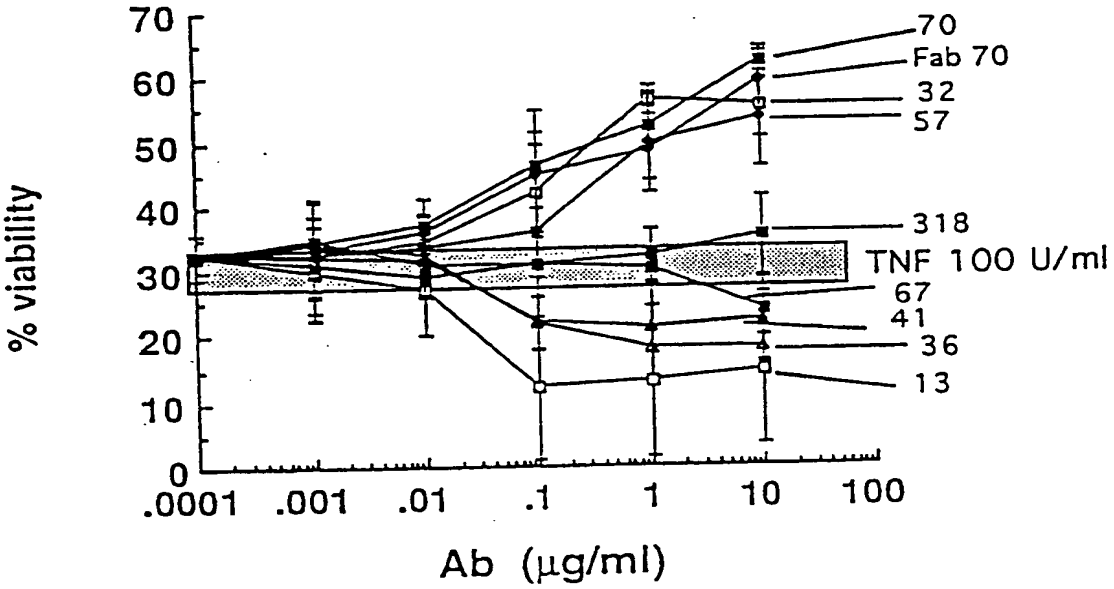

70
60
50
40
30
20
10
0
% viability
.0001
.001
.01
.1
1
10
100
Ab (µg/ml)
70
Fab 70
32
57
318
TNF 100 U/ml
67
41
36
13

FIGURE 6

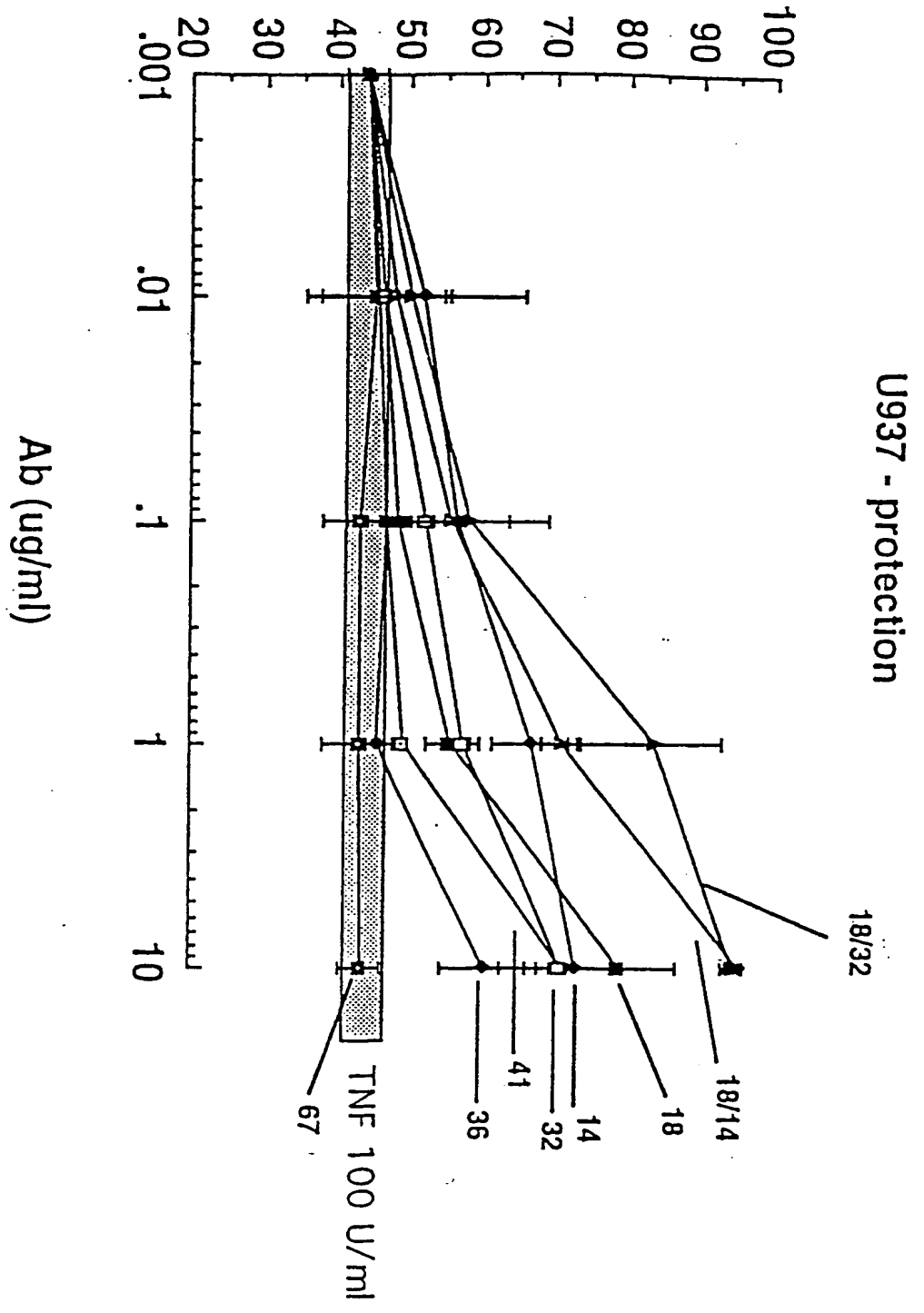

U937 - protection
Ab (ug/ml)
100
90
80
70
60
50
40
30
20
.001
.01
.1
1
10
18/32
18/14
18
14
32
41
36
67
TNF 100 U/ml

FIGURE 7

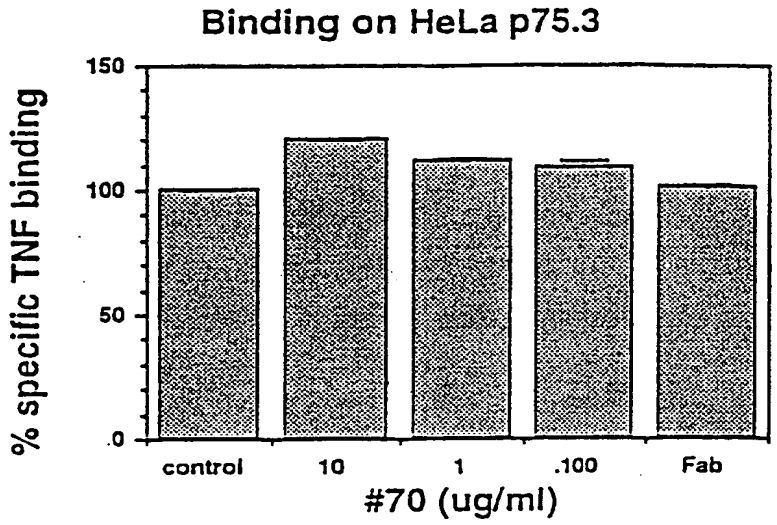


FIGURE 8A

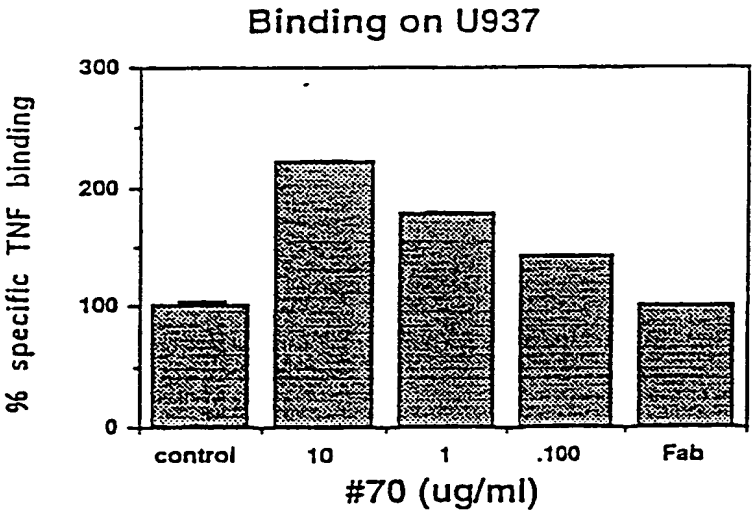
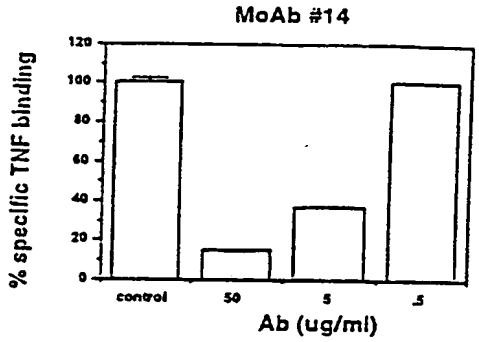
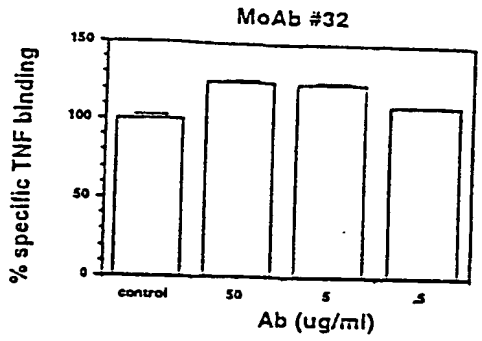
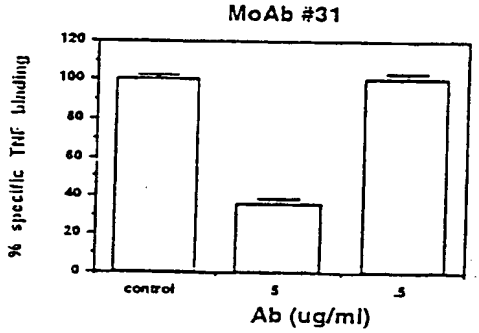
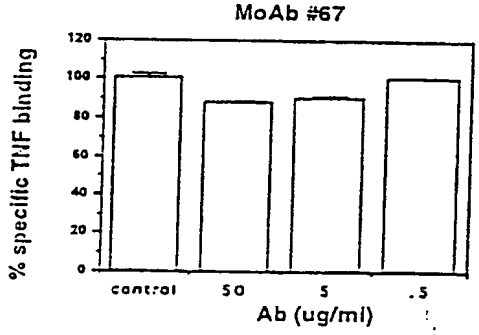


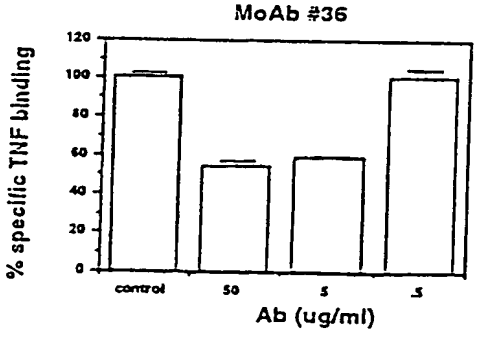
FIGURE 8B


MoAb #14
% specific TNF binding
120
100
80
60
40
20
0
control
50
5
.5
Ab (ug/ml)


MoAb #32
% specific TNF binding
150
100
50
0
control
50
5
.5
Ab (ug/ml)


MoAb #31
% specific TNF binding
120
100
80
60
40
20
0
control
5
.5
Ab (ug/ml)


MoAb #67
% specific TNF binding
120
100
80
60
40
20
0
control
50
5
.5
Ab (ug/ml)


MoAb #36
% specific TNF binding
120
100
80
60
40
20
0
control
50
5
.5
Ab (ug/ml)

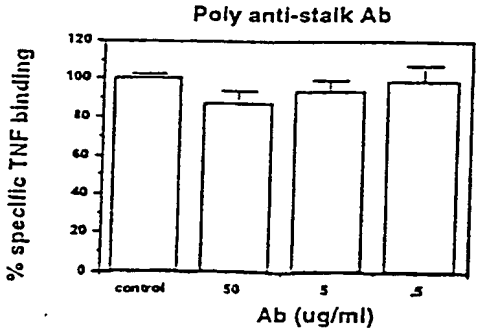

Poly anti-stalk Ab
% specific TNF binding
120
100
80
60
40
20
0
control
50
5
.5
Ab (ug/ml)

FIGURE 9

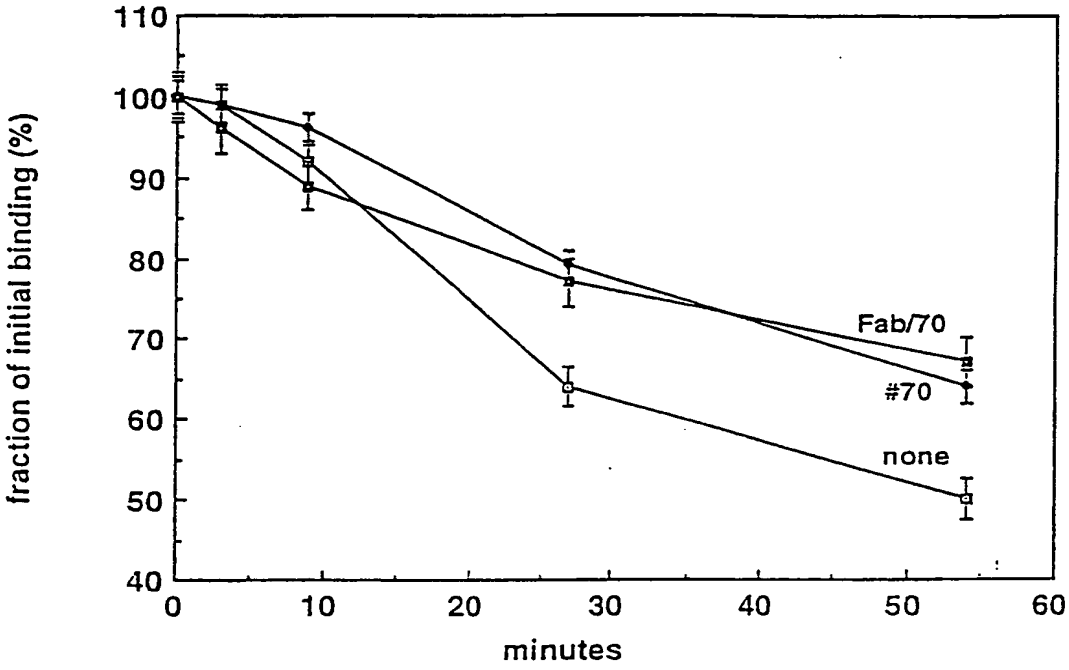

fraction of initial binding (%)
110
100
90
80
70
60
50
40
0
10
20
30
40
50
60
minutes
Fab/70
#70
none

FIGURE 10

```
        1/1                                     31/11
# 70    GTG AAA CTG CAG GAG TCT GGA CCT GAG CTG GTG AAG CCT GGG GCC TCA GTG AAG ATT TCC
        V   K   L   Q   E   S   G   P   E   L   V   K   P   G   A   S   V   K   I   S
        1/1                                     31/11
# 32                                CCT GAG CTG GTG GCT CCT GGG GCC TCA GTG AAG ATT TCC
                                    P   E   L   V   A   P   G   A   S   V   K   I   S
        1/1                                     31/11
# 57    GTG TCC CTG CAG GAG TCT GGG GGA GGC TTA GTG CAG CCT GGA GGG TCC CGG AAA CTC TCC
        V   S   L   Q   E   S   G   G   G   L   V   Q   P   G   G   S   R   K   L   S
--------------------------------------------------------------------------------------

        61/21                                   91/31
# 70    TGC AAA ACT TCT GGC TTC GCA TTC AGT CAT TCT TGG ATG AAC TGG GTG AGG CAG AGG CCT
        C   K   T   S   G   F   A   F   S   H   S   W   M   N   W   V   R   Q   R   P
        61/21                                   91/31
# 32    TGC AAA GCT TCT GGC TAC GCA TTC AGT CAC TCT TGG ATG AAC TGG GTG AAG CAG AGG CCT
        C   K   A   S   G   Y   A   F   S   H   S   W   M   N   W   V   K   Q   R   P
        61/21                                   91/31
# 57    TGT GCA GCT TCT GGA TTC ACT TTC AGT AGC TTT GGA ATG CAC TGG GTT CGT CAG GCT CCA
        C   A   A   S   G   F   T   F   S   S   F   G   M   H   W   V   R   Q   A   P
--------------------------------------------------------------------------------------

        121/41                                  151/51
# 70    GGA CAG GGT CTT GAA TGG ATT GGA CGG ATT TAT CCT GGA GAT GGA AAT ACT GAT TAC CCT
        G   Q   G   L   E   W   I   G   R   I   Y   P   G   D   G   N   T   D   Y   N
        121/41                                  151/51
# 32    GGA AAG GGT CTT GAG TGG ATT GGA CGG ATT CAT CCT GGA GAT GGA GAC ACT GAC TAC AAT
        G   K   G   L   E   W   I   G   R   I   H   P   G   D   G   D   T   D   Y   N
        121/41                                  151/51
# 57    GAG AAG GGG CTG GAG TGG GTC GCA TAC ATT AGT AGT GGC AGT AGT ACC CTC CAC TAT GCA
        E   K   G   L   E   W   V   A   Y   I   S   S   G   S   S   T   L   H   Y   A
--------------------------------------------------------------------------------------

        181/61                                  211/71
# 70    GGG AAG TTC CAG GGC CAG GCC ACA CTG ACT GCA GAC AAA TCT TCC AGC ACA GCC TAC ATG
        G   K   F   Q   G   Q   A   T   L   T   A   D   K   S   S   S   T   A   Y   M
        181/61                                  211/71
# 32    GGG AAC TTC AGG GGC AAG GCC ACA CTG ACT GCA GAC ACA TCC TCC AGC TCA GCC TAC ATG
        G   N   F   R   G   K   A   T   L   T   A   D   T   S   S   S   S   A   Y   M
        181/61                                  211/71
# 57    GAC ACA GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT CCC AAG AAC ACG CTG TTC CTG
        D   T   V   K   G   R   F   T   I   S   R   D   N   P   K   N   T   L   F   L
--------------------------------------------------------------------------------------

        241/81                                  271/91
# 70    CAA CTC TTC AGT CTG ACC TCT GTG GAC TCT GCG GTC TAT TTT TGT GCA CCC GGC CGT TGG
        Q   L   F   S   L   T   S   V   D   S   A   V   Y   F   C   A   P   G   R   W
        241/81
# 32    CAG CTC AGC AGC CTG ACC TCT GTG GAT TCT GCG GTC TAC TTC TGT GCA CCC GGC CGT TGG
        Q   L   S   S   L   T   S   V   D   S   A   V   Y   F   C   A   P   G   R   W
        241/81                                  271/91
# 57    CAA ATG AAA CTA CCC TCA CTA TGC TAT GGA CTA CTG GGG CCA AGG GAC CAC GGT CAC CGT
        Q   M   K   L   P   S   L   C   Y   G   L   L   G   P   R   D   H   G   H   R
--------------------------------------------------------------------------------------

        301/101                                 331/111
# 70    TAC CTC GAA GTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
        Y   L   E   V   W   G   Q   G   T   T   V   T   V   S   S
        301/101
# 32    TAC CTC GAG GTC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
        Y   L   E   V   W   G   Q   G   T   T   V   T   V   S   S
        301/101
# 57    CTC CTC A
        L   L
```

FIGURE 11

```
                                                                31/11
                                                TCC TCC CTG GCT ATG TCA GTA GGA CAG ATG GTC ACT
                                                S   S   L   A   M   S   V   G   Q   M   V   T
61/21                                                           91/31
ATG AGC TGC AAG TCC AGT CAG AGC CTT TTA ACT AGT AGC ACT CAA AAG AAC TCT TTG GCC
M   S   C   K   S   S   Q   S   L   L   T   S   S   T   Q   K   N   S   L   A
121/41                                                          151/51
TGG TAC CAG CAG ACA CCA GGA CAG TCT CCT AAA CTT CTG ATA TAC TTT GCA TCC ACT AGG
W   Y   Q   Q   T   P   G   Q   S   P   K   L   L   I   Y   F   A   S   T   R
181/61                                                          211/71
CTA TCT GGG GTC CCT GAT CGC TTC ATA GGC AGT GGA TCT GGG ACA GAT TTC ACT CTT ACC
L   S   G   V   P   D   R   F   I   G   S   G   S   G   T   D   F   T   L   T
241/81                                                          271/91
ATC AGC AGT GTG CAG GCT GAA GAC CTG GCA GAT TAC TTC TGT CAG CAA CAT TAT AGC ACT
I   S   S   V   Q   A   E   D   L   A   D   Y   F   C   Q   Q   H   Y   S   T
301/101                                                         331/111
CCA TTT ACG TTC GGC TCG GGG ACA AAG TTG GAA ATA GAG CGG GCT GAT GCT GCA CCA ACT
P   F   T   F   G   S   G   T   K   L   E   I   E   R   A   D   A   A   P   T
361/121
GTA TCC ATC TTC CCA CCA TCC A
V   S   I   F   P   P   S
```

FIGURE 12

```
hu p55 TNF-R  (3-42)
hu p75 TNF-R  (39-76)
hu FAS        (31-67)
hu NGF-R      (3-37)
hu CDw40      (25-60)
rat Ox40      (25-60)

hu p55 TNF-R  (41-86)
hu p75 TNF-R  (77-119)
hu FAS        (68-112)
hu NGF-R      (30-80)
hu CDw40      (61-104)
rat Ox40      (61-104)

hu p55 TNF-R  (87-126)
hu p75 TNF-R  (120-162)
hu FAS        (113-149)
hu NGF-R      (81-119)
hu CDw40      (105-144)
rat Ox40      (105-123)

hu p55 TNF-R  (127-155)
hu p75 TNF-R  (163-201)
hu NGF-R      (120-161)
hu CDw40      (145-187)
rat Ox40      (124-164)
```

FIGURE 13